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A selenium-based cysteine surrogate for protein chemical synthesis

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We provide in this protocol detailed procedures for the synthesis of SetCys cysteine surrogate and its use for chemical synthesis of proteins through the redox-controlled assembly of three peptide segments in one-pot.

Keywords

N-(2-selenoethyl)cysteine, cysteine surrogate, native chemical ligation (NCL), *bis*(2-sulfanylethyl)amido (SEA)-mediated ligation, chemical protein synthesis, sulfur, selenium, one-pot.

1. Introduction

Modern chemical protein synthesis is usually achieved by the concatenation of unprotected peptide segments through the application of chemoselective peptide bond-forming reactions. Considered as a figurehead in the field, the native chemical ligation (NCL) reaction involves the coupling of a peptide thioester with a cysteinyl (Cys) peptide in aqueous media under neutral conditions. [1,2,3,4] Many target proteins exceed 100 amino acids and are thus too long to be accessed by the coupling of just two peptide segments, if the latter have to be produced by solid phase peptide synthesis (SPPS). [5] The access to challenging protein targets usually requires the resort to two or more NCL reactions, [6] and thus potentially to set up a large number of chemical and isolation steps. In this context and logically, the field of chemical protein synthesis has encountered a growing interest for economy-oriented synthetic approaches [7], which are sustainable as well as time and cost effective by increasing the yield of isolated material through the reduction of intermediate purification steps. To achieve such an objective, solid-phase protein assembly techniques were developed in which elongated polypeptides are simply separated at each step of the process from the reagents and by-products by washing of the solid phase [6,8,9]. An attractive alternative to access large proteins is to use one-pot multi-segment concatenation strategies [6,10]. However, the application of such strategies assumes to have temporarily silent and activatable thioester and/or cysteine surrogates at disposal, so as to trigger ligations in a specific order and to ensure that peptide segments get connected in the proper way.

The *bis*(2-sulfanylethyl)amido (SEA)-mediated ligation is an NCL-related process involving a peptide equipped with a latent thioester moiety in the form of a cyclic disulfide (SEA^{off} peptide

in Figure 1A,B) [11,12].While the SEA^{off} group is unaffected under weak reducing conditions (Figure 1A), the addition of a strong reductant such as *tris*(2-carboxyethyl)phosphine (TCEP) in the *milieu* triggers its ring-opening by reduction of the disulfide bond and its reaction with Cys peptides to yield a ligated product (Figure 1B). Recently, we have developed a cysteine surrogate called SetCys whose reactivity is also dependent on the reducing power of the medium (Figure 1C,D) [13]. The SetCys amino acid is a cyclic selenosulfide analogue of cysteine, which under mild reducing conditions does not interfere with a classical NCL reaction (Figure 1C). In contrast, subjecting SetCys to strong reducing conditions results in the spontaneous loss of its *N*-selenoethyl appendage and thus to its conversion into an N-terminal Cys residue (Figure 1D). If a peptide thioester segment is present in the reacting mixture, the conversion of SetCys to Cys followed by a classical NCL leads to the production of a native polypeptide of larger size (Figure 1D).

[Insert Figure 1 near here]

Figure 1 clearly shows the parallel between SEA^{off} and SetCys regarding their behavior toward the reducing power of the medium. This protocol article illustrates how the redox-controllable SEA^{off} and SetCys groups can be exploited hand-in-hand for the synthesis of proteins through a one-pot three-segment assembly process (Figure 2). The synthesis involves SEA^{off} peptide **1**, the bifunctional peptide **2** featuring a SetCys residue at its N-terminus and a C-terminal alkyl thioester, and finally a Cys peptide **3**. By virtue of the properties presented for each group in Figure 1, the elongation of the polypeptide chain takes place in the C-to-N direction. Indeed, since weakly reducing conditions are first applied, the SEA^{off} and the SetCys groups remain silent. Therefore, partners **2** and **3** first react by classical NCL to form the elongated intermediate **4**. Addition of TCEP and sodium ascorbate (see later) in the reaction mixture and a slight pH-adjustment provide the conditions to activate the SEA^{off} and SetCys groups and thus, an event that triggers the coupling of intermediate polypeptide **4** to SEA peptide **1** with the concomitant conversion of SetCys into Cys. The simultaneous presence of three peptide segments in the reaction mixture from the beginning of the one-pot process is an hallmark of this approach, which is illustrated by the chemical synthesis of a biotinylated analogue of hepatocyte growth factor (HGF) K1 domain (Figure 2).

[Insert Figure 2 near here]

In addition to the assembly of the K1 domain, the detailed synthesis of a conveniently Fmoc-protected SetCys amino acid **6** for solid phase peptide synthesis as well as its incorporation into bifunctional peptides are described hereafter.

2. Materials

2.1 Health and safety recommendations

All organic solvents and reagents used in this protocol should be handled inside a chemical fume hood with appropriate personal protective equipment (lab coat, gloves and protective glasses). Solvents were removed using a rotary evaporator placed inside a fume hood. Particular care must be taken for the manipulation of selenium containing reagents and synthetic intermediates due to their potential toxicity. Trifluoroacetic acid is strongly corrosive and toxic. Therefore, it should be handled with the greatest attention.

2.2 General laboratory equipments, solvents and reagents

2.2.1 General materials for organic synthesis and peptide chemistry

1. Conventional laboratory glassware.
2. Microfuge tubes (0.5, 1.5 mL and 5 mL safe-lock tubes; Eppendorf), plastic tubes (15 mL and 50 mL) for centrifugation.
3. Glove box (Jacomex) equipped with a block heater (37°C), a set of adjustable pipettes (0.5–10, 2–20, 10–50, 10–100, 100–1,000 µL, Eppendorf), corresponding pipette tips, a pH meter (Eutech instrument) and a vortex shaker.
4. Argon gas (O_2 < 0.01 ppm, H_2O < 0.02 ppm, Air Liquide).
5. Rotatory evaporator.
6. TLC UV Cabinet (254 nm).
7. TLC plates (Silica gel 60 F_{254} on aluminium sheets).
8. Deionized water (≥ 18 MΩ.cm).
9. High purity grade solvents for classical organic synthesis and peptide chemistry: acetonitrile, chloroform ($CHCl_3$), cyclohexane (Cy), 1,2-dichloroethane (DCE), dichloromethane (DCM), diethyl ether (Et_2O), *N*,*N*-dimethylformamide (DMF) for peptide synthesis, dioxane, ethanol (EtOH), ethyl acetate (EtOAc), *n*-heptanes, *n*-hexanes, methanol (MeOH).
10. *N*,*N*-diisopropylethylamine for peptide synthesis (DIEA).
11. Guanidine hydrochloride (Gn·HCl).

12. High purity grade reagents for classical organic synthesis and peptide chemistry: sodium bicarbonate ($NaHCO_3$), sodium chloride (NaCl), potassium carbonate (K_2CO_3), hydrochloric acid (HCl, 37%), sodium hydroxide (NaOH), anhydrous magnesium sulfate ($MgSO_4$).
13. Silica gel (60-200 µm, 60 Å).

2.2.2 HPLC analysis & purification

2.2.2.1. Analytical UPLC-MS analysis

1. Analytical UPLC–MS analyses were performed on Dionex Ultimate 3000 UHPLC system (Thermofisher) equipped with an Acquity UPLC Peptide BEH300 C18 reverse phase-column (2.1 × 100 mm, pore size: 300 Å, particle size: 1.7 µm), a diode array detector, a charged aerosol detector (CAD) and a mass spectrometer (Ion trap LCQfleet).
2. MS-grade trifluoroacetic acid.
3. LC-MS grade acetonitrile (ACN).
4. Eluant A for UPLC-MS analysis: TFA 0.1% (vol/vol) in deionized water. Add 2.5 mL of TFA in 2.5 L of deionized water.
5. Eluant B for UPLC-MS analysis: TFA 0.1% (vol/vol) in LC-MS grade ACN. Add 2.5 mL of TFA in 2.5 L of ACN.
6. Standard method for analyzing peptides by UPLC-MS. Gradient: 0-70% eluent B in 20 min, flow rate: 0.4 mL/min, temperature: 50 °C, UV detection at 215 nm.

2.2.2.2. Preparative HPLC

1. Preparative HPLC were performed on a PLC2020 Gilson system equipped with a C18 reverse-phase column.
   - for small quantities of crude peptide (< 20 mg): Waters XBridge BEH300 C18 reverse-phase column (10 × 250 mm; pore size: 300 Å; particle size: 5 µm).
   - for large quantities of crude peptide (> 20 mg): Waters XBridge Prep C18 reverse-phase column (19 × 150 mm; pore size: 130 Å, particle size: 5 µm).
2. UV-grade trifluoroacetic acid.
3. HPLC-grade ACN
4. TFA 10% (vol/vol) in deionized water. Add 100 mL of TFA carefully to 900 mL of deionized water.

5. Eluent A for semi-preparative HPLC. TFA 0.1% (vol/vol) in deionized water. Add 10 mL of TFA 10% (vol/vol) in water to 990 mL of deionized water.
6. Eluent B for semi-preparative HPLC. TFA 0.1% (vol/vol) in ACN. Add 10 mL of 10% aqueous TFA (vol/vol) to 990 mL of ACN.

2.2.3 Lyophilization

1. Lyophilizer (Christ Gamma 2-20) equipped with a manifold.
2. Lyophilizer flasks (VWR, 300 mL).
3. Liquid nitrogen.
4. Dewar.

2.2.4 MALDI-TOF analysis

1. MALDI-TOF mass spectrometer (Autoflex Speed, Bruker).
2. α-Cyano-4-hydroxycinnamic acid.
3. 2,5-Dihydroxybenzoic acid.
4. A solution of α-cyano-4-hydroxycinnamic acid (10 mg/mL) in 50% aqueous ACN containing 0.1% TFA.
5. A solution of 2,5-dihydroxybenzoic acid (16 mg/mL) in 50% aqueous ACN containing 0.1% TFA.
6. To perform MALDI-TOF analysis, mix 1 µL of α-cyano-4-hydroxycinnamic acid solution or 2,5-dihydroxybenzoic acid solution with 1 µL of the sample on the MALDI plate. Let dry at room temperature in air prior to analysis.

2.2.5 NMR analysis

1. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Advance-300 spectrometer operating at 300 and 75 MHz, respectively. The spectra are reported as parts per million (ppm) down field shift using tetramethylsilane as internal reference. The data are reported as chemical shift (δ), multiplicity, relative integral, coupling constant (J Hz), and assignment when possible.

2.2.5 Polarimetric analysis

1. Optical rotations were recorded at 20 °C on a Perkin-Elmer 343 digital polarimeter at 589 nm with a cell path length of 10 cm.

2.2.6. HRMS analysis

1. For HRMS analyses, this work has benefited from the facility and expertise of "Service HPLC-MASSE" (Institut de Chimie des Substances Naturelles, Centre de Recherche de Gif sur Yvette, CNRS, 91198, Gif sur Yvette, France).

2.3 Synthesis of the Fmoc-SetCys-OH amino acid 6

2.3.1 Synthesis of *bis*(2,2-diethoxyethyl)diselenide 8

1. Potassium selenocyanate (KSeCN).
2. 2-bromo-1,1-diethoxyethane (**7** in Figure 3).

2.3.2 Synthesis of (2,2-diethoxyethyl)(4-methoxybenzyl)selenide 9

1. Sodium borohydride ($NaBH_4$).
2. 4-methoxybenzyl chloride (PMBCl).

2.3.3 Synthesis of 2-((4-methoxybenzyl)selenyl)acetaldehyde 10

1. Formic acid. A 1 M aqueous solution is prepared by adding formic acid (0.37 mL, 10 mmol) in a 10 mL volumetric flask and completing with deionized water. Mix the flask thoroughly before use.

2.3.4 Synthesis of H-Cys(Trt)-OMe 12

1. Cysteine methyl ester hydrochloride (**11** in Figure 3).
2. Trifluoroacetic acid (TFA).
3. Triphenylmethanol.
4. The 0.25 M aqueous solution of K_2CO_3 is prepared by dissolving K_2CO_3 (3.45 g, 25.0 mmol) in deionized water (100 mL).

2.3.5 Synthesis of *N*-[PMBSe-$(CH_2)_2$]-Cys(Trt)-OMe 13

1. Powdered 3 Å molecular sieves.
2. Sodium triacetoxyborohydride ($NaBH(OAc)_3$).
3. The 0.5 M aqueous solution of K_2CO_3 is prepared by dissolving K_2CO_3 (6.90 g, 50.0 mmol) in water (100 mL).

2.3.6 Synthesis of *N*-Fmoc-*N*-[PMBSe-$(CH_2)_2$]-Cys(Trt)-OMe 14

1. 9-Fluorenylmethoxycarbonyl chloride (Fmoc-Cl).

2.3.7 Synthesis of Fmoc-SetCys-OMe 15

1. Iodine (I_2).
2. Sodium sulfate pentahydrate ($Na_2S_2O_3 \cdot 5\ H_2O$). An aqueous solution of $Na_2S_2O_3$ is prepared by dissolving $Na_2S_2O_3 \cdot 5\ H_2O$ (24.8 g, 100 mmol) in water (100 mL).

2.3.8 Synthesis of Fmoc-SetCys-OH 6

1. ~5 M HCl. The solution is prepared by adding HCl (37%, 8.3 mL) to deionized water (11.7 mL) in a graduated cylinder.

2.4 Synthesis of the bifunctional peptide 2 (SetCys-HGF 150-176-MPA)

1. SEA-PS (polystyrene) solid support, loading: 0.16 mmol/g (prepared according to ref [11]).
2. 1-Hydroxybenzotriazole hydrate (HOBt).
3. *N*,*N*'-Diisopropylcarbodiimide (DIC).
4. Acetic anhydride (Ac_2O).
5. Piperidine.
6. Capping mixture (SPPS): 10 % Ac_2O, 5 % DIEA in DMF (vol/vol/vol). Add 2 mL of Ac_2O and 1 mL of DIEA to 17 mL of DMF.
7. Piperidine solution for Fmoc removal (SPPS): 20% piperidine in DMF (vol/vol). Add 4 mL of piperidine in 16 mL of DMF.
8. Triisopropylsilane (TIS).
9. Thioanisole.
10. Thiophenol.
11. 3-Mercaptopropionic acid (MPA).

2.5 One-pot C-to-N three peptide segment assembly

1. 4-Mercaptophenylacetic acid (MPAA).
2. Disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4 \cdot 12H_2O$).
3. Sodium dihydrogen phosphate dihydrate ($NaH_2PO_4 \cdot 2H_2O$).
4. 0.1 M sodium phosphate buffer pH 7.2. Prepare 36 mL of a 0.2 M solution of disodium hydrogen phosphate in deionized water. Prepare 10 mL of a 0.2 M solution of sodium

dihydrogen phosphate in deionized water. Mix the two solutions and verify the pH. Dilute the prepared sodium phosphate buffer two-fold prior use.

5. *Tris*(2-carboxyethyl)phosphine hydrochloride (TCEP·HCl).
6. Sodium ascorbate.
7. Glacial acetic acid (AcOH).

3 Methods

2.1 Synthesis of the Fmoc-SetCys-OH amino acid 6

The synthetic strategy proposed for the preparation of the Fmoc-protected SetCys amino acid **6** involves a convergent approach with a total of five steps from the affordable and commercially available cysteine methyl ester hydrochloride **11** (Figure 3). After the protection of Cys thiol side chain, intermediate **12** undergoes a reductive alkylation to install the selenated appendage on the α-amino group. Amine protection by a Fmoc group is followed by a tandem oxidative deprotection/cyclization process to afford the cyclic selenosulfide **15**. Final liberation of the acid moiety is achieved by HCl-mediated hydrolysis to provide Fmoc-SetCys-OH **6** with an overall yield of 34% (*see* **Notes §1** and **2**).

[Insert Figure 3 near here]

3.1.1 Synthesis of *bis*(2,2-diethoxyethyl)diselenide 8

Diselenide **8** was prepared by a procedure similar to that reported by Krief *et al.* [14]

1. In a two-neck flask equipped with a septum and a reflux condenser and maintained under argon atmosphere, dissolve potassium selenocyanate (576 mg, 4.00 mmol) in DMF (10 mL). The resulting solution turns to a clear yellowish color.
2. Place the flask in an oil bath and heat the mixture at 75 °C (bath temperature). Introduce dropwise by syringe a solution of 2-bromo-1,1-diethoxyethane **7** (4.00 mmol, 601 µL) in DMF (4 mL). Stir the reaction mixture at 75 °C for 3 h. The solution becomes brownish.
3. Add dropwise by syringe a solution of K_2CO_3 (552 mg, 4.00 mmol) in deionized water (1.6 mL). Keep stirring the reaction mixture for 3 h at 75 °C.
4. *Workup*. Remove the oil bath, add at RT 20 mL of deionized water and transfer the mixture to a separatory funnel. Extract the aqueous layer with Et_2O (2 × 20 mL) (*see* **Note §3**), wash the combined organic layers with water (2 × 20 mL) and dry over

anhydrous $MgSO_4$. Filter and evaporate the solvent to dryness under reduced pressure in a rotatory evaporator. Purify the crude product by column chromatography on silica gel (hexane/EtOAc 95:5) to obtain diselenide **8** as a yellow oil (485 mg, 62%).

5. Check the identity and purity of the synthesized compound by NMR. **^{1}H NMR** (300 MHz, $CDCl_3$, 298 K) δ 4.70 (t, *J* = 5.7 Hz, 2H), 3.63–3.73 (m, 4H), 3.52–3.61 (m 4H), 3.22 (d, *J* = 5.7 Hz, 4H), 1.22 (t, *J* = 7.1 Hz, 12H).

3.1.2 Synthesis of (2,2-diethoxyethyl)(4-methoxybenzyl)selenide 9

Selenide **9** was prepared by a procedure similar to that reported by Abbas *et al.* [15]

1. In round-bottom flask maintained under an argon atmosphere, dissolve diselenide **8** (900 mg, 2.29 mmol) in absolute ethanol (14 mL).
2. Place the flask in an ice bath and add $NaBH_4$ (175 mg, 4.62 mmol) portionwise under a stream of argon gas (*see* **Note §4**). Remove the ice bath and allow the mixture to stir at RT for 30 min. A white precipitate initially forms and redissolves resulting in a homogeneous solution.
3. Place again the flask in an ice bath and add 4-methoxybenzyl chloride (561 µL, 4.14 mmol) dropwise. A white precipitate forms. Remove the ice bath and stir the reaction mixture for 3 h at RT.
4. *Workup.* Filter the crude suspension through a celite pad in order to remove the white precipitate. Concentrate the clear yellowish filtrate under reduced pressure in a rotatory evaporator and add 30 mL of water. Transfer the aqueous mixture to a separatory funnel and extract it with Et_2O (2 × 25 mL). Dry the combined organic layers over $MgSO_4$, filter and evaporate the solvent under reduced pressure in a rotatory evaporator. Purify the crude product by silica gel column chromatography (hexanes/EtOAc 95:5) to obtain selenide **9** as a yellow oil (1.19 mg, 90%).
5. Check the identity and purity of the synthesized compound by ^{1}H NMR. **^{1}H NMR** (300 MHz, $CDCl_3$, 298 K) δ 7.23 (d, *J* = 8.6 Hz, 2H), 6.82 (d, *J* = 8.6 Hz, 2H), 4.59 (t, *J* = 5.6 Hz, 1H), 3.82 (s, 2H), 3.79 (s, 3H), 3.59–3.70 (m, 2H), 3.46–3.56 (m, 2H), 2.62 (d, J = 5.6 Hz, 2H), 1.22 (t, *J* = 7.1 Hz, 6H).

3.1.3 Synthesis of 2-((4-methoxybenzyl)selenyl)acetaldehyde 10

Aldehyde **10** was prepared by a procedure similar to that reported by Abbas *et al.* [15]

1. In a round-bottom flask equipped with a reflux condenser, suspend acetal **9** (1.78 g, 5.61 mmol) in 1 M aqueous formic acid solution (24 mL).

2. Place the flask in an oil bath and heat the mixture overnight at 50 °C (oil bath temperature).
3. *Workup*. Remove the oil bath and add water (55 mL) at RT. Transfer the aqueous mixture to a separatory funnel and extract it with Et_2O (2 × 55 mL). Wash the combined organic layers with water (40 mL) and dry the organic solution over $MgSO_4$. Filter and evaporate the solvent under reduced pressure in a rotatory evaporator. Aldehyde **10** (838 mg, 61%) is directly used in the next step without further purifications (*see* **Note §5**).
4. Check the identity and purity of the synthesized compound by ^{1}H NMR. **^{1}H NMR** (300 MHz, $CDCl_3$, 298 K) δ 9.36 (t, *J* = 4.2 Hz, 1H), 7.22 (d, *J* = 8.7 Hz, 2H), 6.84 (d, *J* = 8.7 Hz, 2H), 3.79 (s, 3H), 3.64 (s, 2H), 3.12 (d, *J* = 4.1 Hz, 2H).

3.1.4 Synthesis of H-Cys(Trt)-OMe 12

1. In a round-bottom flask, dissolve under argon cysteine methyl ester hydrochloride **11** (856 mg, 4.99 mmol) in TFA (5 mL).
2. Add triphenylmethanol (1.43 g, 5.49 mmol) and stir the mixture at RT for 5 h. The solution turns brown upon addition of triphenylmethanol.
3. Evaporate the TFA under reduced pressure in a rotatory evaporator and take up the residue in MeOH (27 mL). Keep on stirring at RT until the yellow color disappears.
4. Evaporate MeOH under reduced pressure in a rotatory evaporator and suspend the residue in a 0.25 M K_2CO_3 aqueous solution (30 mL) (*see* **Notes §6** and **7**).
5. *Workup*. Transfer the aqueous mixture in a separatory funnel and extract it with Et_2O (2 × 20 mL). Dry the combined organic layers over $MgSO_4$, filter and evaporate the solvent under reduced pressure in a rotatory evaporator. Purify the crude product by silica gel column chromatography (DCM/MeOH 98:2) to obtain the trityl-protected cysteine **12** (1.73 g, 92%) as a viscous oil.
6. Check the identity and purity of the synthesized compound by NMR. **^{1}H NMR** (300 MHz, $CDCl_3$, 298 K) δ 7.41-7.44 (m, 6H), 7.18-7.31 (m, 9H), 3.65 (s, 3H), 3.20 (dd, *J* = 4.8 and 7.8 Hz, 1H), 2.59 (dd, *J* = 4.8 and 12.4 Hz, 1H), 2.46 (d, *J* = 7.8 and 12.4 Hz, 1H) ppm. **^{13}C NMR** (75 MHz, $CDCl_3$, 298 K) δ 174.3 (C), 144.6 (3 × CH), 129.7 (6 × CH), 128.1 (6 × CH), 126.9 (3 × CH), 66.9 (C), 53.9 (CH), 52.3 (CH_3), 37.2 (CH_2) ppm.

3.1.5 Synthesis of *N*-[PMBSe-(CH_2)$_2$]-Cys(Trt)-OMe 13

4. In a round-bottom flask, dissolve under argon aldehyde **10** (838 mg, 3.45 mmol) in DCE (50 mL).

5. To this solution, successively add ≈ 4 g of activated powdered 3 Å molecular sieve (*see* **Note §8**), sodium triacetoxyborohydride (1.02 g, 4.81 mmol) and a solution of trityl-protected cysteine **12** (1.30 g, 3.44 mmol) in DCE (35 mL).
6. After 18 h of stirring at RT, check the completion of the reaction by TLC.
7. *Workup*. Filter the reaction mixture on a Büchner funnel and wash the solid with additional portions of DCM. Concentrate the filtrate under reduced pressure in a rotatory evaporator and take up the residue in a 0.5 M aqueous K_2CO_3 solution (50 mL). Transfer the aqueous mixture to a separatory funnel and extract it with DCM (3 × 50 mL). Dry the combined organic layers over $MgSO_4$, filter and evaporate the solvent under reduced pressure in a rotatory evaporator. Purify the crude by silica gel column chromatography (Cy/EtOAc 80:20) to obtain *N*-alkylated cysteine **13** as a clear yellow oil (1.17 g, 56%).
8. Check the identity and purity of the synthesized compound. **^{1}H NMR** (300 MHz, $CDCl_3$, 298 K) δ 7.41 (d, *J* = 7.4 Hz, 6H), 7.19-7.32 (m, 9H), 7.17 (d, *J* = 8.6 Hz, 2H), 6.79 (d, *J* = 8.6 Hz, 2H), 3.77 (s, 3H), 3.71 (s, 2H), 3.66 (s, 3H), 2.99 (t, *J* = 6.5 Hz, 1H), 2.37-2.74 (m, 6H), 1.44-1.91 (m, 1H) ppm. **^{13}C NMR** (75 MHz, $CDCl_3$, 298 K) δ 173.5 (C), 158.4 (C), 144.6 (3 × C), 131.1 (C), 129.9 (2 × CH), 129.6 (6 × CH), 127.9 (6 × CH), 126.7 (3 × CH), 113.9 (2 × CH), 66.8 (C), 60.2 (CH), 55.2 (CH_3), 51.9 (CH_3), 47.3 (CH_2), 34.6 (CH_2), 26.3 (CH_2), 23.9 (CH_2) ppm. **HRMS** (ES^+): Calcd. for $C_{33}H_{35}NO_3SNaSe$: 628.1401, found: 628.1378. **$[\alpha]_D^{20}$** -19 ° (*c* 1.0, chloroform).

3.1.6 Synthesis of *N*-Fmoc-*N*-[PMBSe-(CH_2)$_2$]-Cys(Trt)-OMe 14

1. In a round-bottom flask maintained under argon, dissolve compound **13** (455 mg, 0.752 mmol) in DCM (3.75 mL)
2. Successively add DIEA (0.137 mL, 0.786 mmol) and Fmoc-Cl (204 mg, 0.788 mmol).
3. Stir the reaction mixture at RT for 24 h.
4. *Workup*. Dilute the reaction mixture with DCM (20 mL). Transfer the DCM solution to a separatory funnel and wash it with water (2 × 20 mL). Dry the organic layer over $MgSO_4$, filter and evaporate the solvent under reduced pressure in a rotatory evaporator. Purify the crude mixture by silica gel column chromatography (Cy/EtOAc 7:3) to obtain Fmoc-protected compound **14** as a white solid (522 mg, 84%).
5. Check the identity and purity of the synthesized compound. **^{1}H NMR** (300 MHz, $CDCl_3$, 298 K) and **^{13}C NMR** (75 MHz, $CDCl_3$, 298 K). Use the provided NMR FID files for compound **14** to make comparisons (*see* **Notes §9** and **10**). **HRMS** (ES^+): Calcd. for $C_{48}H_{45}NO_5SNaSe$: 850.2081, found: 850.2089. **$[\alpha]_D^{20}$** (c 1.0, $CHCl_3$): -38 °.

3.1.7 Synthesis of Fmoc-SetCys-OMe 15

1. In a round-bottom flask maintained under argon, dissolve compound **14** (1.67 g, 2.02 mmol) in DCM (50 mL) and add $NaHCO_3$ (508 mg, 6.06 mmol).
2. Place the flask in an ice bath and add dropwise by syringe a solution of I_2 (1.54 g, 6.06 mmol) in DCM (50 mL).
3. Remove the ice bath and stir the reaction mixture at RT for 30 min. Check the complete consumption of the starting material by TLC (*see* **Note §11**).
4. *Workup*. Add 1 M $Na_2S_2O_3$ (50 mL) to remove the excess of I_2. Separate the organic and aqueous phases in a separatory funnel (*see* **Note 12**) and extract the aqueous layer with DCM (2 × 50 mL). Wash the combined DCM layers layer with brine (50 mL) and dry over $MgSO_4$. Filter and concentrate the solvent under reduced pressure in a rotatory evaporator (*see* **Note §13**). Purify the crude by silica gel column chromatography (Cy/EtOAc 8:2) (*see* **Note §14**) to obtain Fmoc-SetCys-OMe **15** as a colorless glass (780 mg, 83%).
5. Check the identity and purity of the synthesized compound. **^{1}H NMR** (300 MHz, $CDCl_3$, 298 K) and **^{13}C NMR** (75 MHz, $CDCl_3$, 298 K). Use the provided NMR FID files for compound **15** to make comparisons (*see* **Notes §9** and **10**). **HRMS** (ES^+) Calcd. for $C_{21}H_{21}NO_4NaSSe$: 486.0254, found: 486.0246. **$[\alpha]_D^{20}$** (c 1.0, $CHCl_3$): -29 °.

3.1.8 Synthesis of Fmoc-SetCys-OH 6

1. In a round-bottom flask equipped with reflux condenser and maintained under argon, dissolve compound **15** (532 mg, 1.15 mmol) in dioxane (22 mL).
2. Add 5 M HCl (11 mL).
3. Place the flask in an oil bath and heat the mixture for 12 h (overnight) at 80 °C (oil bath temperature).
4. Remove the oil bath and add at RT a 5% aqueous K_2CO_3 solution (200 mL) in order to raise the pH above 9 (*see* **Note §6**).
5. *Workup*. Transfer the mixture to a separatory funnel and wash it with Et_2O (2 × 100 mL). Acidify the aqueous layer with 12 N HCl until pH ≈ 2-3 (*see* **Note §6**) and extract it with DCM (3 × 100 mL). Dry the combined DCM layers over $MgSO_4$, filter and evaporate the solvent under reduced pressure in a rotatory evaporator. Fmoc-SetCys-OH **6** is isolated as a light yellow solid (488 mg, 95%) and is pure enough to be used in Fmoc-SPPS without further purification.

6. Check the structure and purity of the synthesized compound. **^{1}H NMR** (300 MHz, $CDCl_3$, 298 K) and **^{13}C NMR** (75 MHz, $CDCl_3$, 298 K). Use the provided NMR FID files for compound **6** to make comparisons (*see* **Notes §9** and **10**). **HRMS** (ES^+): Calcd. for $C_{20}H_{19}NO_4NaSSe$: 472.0098, found: 472.0116. **$[\alpha]_D^{20}$** (c 1.0, $CHCl_3$): -32 °.

3.2 Synthesis of the bifunctional peptide 2 (SetCys-HGF 150-176-MPA)

The incorporation of Fmoc-SetCys-OH residue **6** at the N-terminus of a bifunctional peptide 2 is achieved as described in Figure 4. The peptide is first elongated on a SEA-functionalized solid support using an automated peptide synthesizer. Fmoc-SetCys-OH **6** is then manually coupled to the elongated peptide using DIC/HOBt activation. After Fmoc removal, the peptide is cleaved from the solid support, liberating a bifunctional N-terminal SetCys and a C-terminal SEA^{on} group. The latter is immediately subjected to a SEA / 3-mercaptopropionic (MPA) exchange reaction in the absence of TCEP to avoid any opening of the SetCys group by reduction of the selenosulfide bond. The whole detailed procedure is described below.

[Insert Figure 4 near here]

Elongation of the peptide on SEA-functionalized solid support:

1. Elongate the HGF 150-176 peptide segment by Fmoc-SPPS (0.05 mol scale) on a SEA-functionalized solid support (loading 0.16 mmol/g) using the protocol described in ref [16]. At the end of the automated process, the peptidyl solid support is in suspension in DCM.
2. Transfer the peptidyl solid support obtained in step 1 into a glass reactor for manual solid phase synthesis equipped with a fritted glass and a stopcock at its bottom. Drain the DCM.
3. Wash the beads with DMF (3 × 10 mL) and drain.
4. Swell the peptidyl solid support in the minimal volume of DMF.
5. In a 5-mL microfuge tube, dissolve the SetCys amino acid **6** (44.8 mg, 0.100 mmol) in DMF (2.5 mL). Successively add HOBt (15.3 mg, 0.100 mmol) and DIC (15.6 µL, 0.100 mmol) and stir the mixture for 2 min.
6. Add the pre-activated amino acid (from step 5) to the reactor containing the solid support. Shake the mixture on a wrist-action shaker for 3 h.
7. Wash the beads with DMF (3 × 10 mL) and drain (*see* **Note §15**).

8. Add 10 mL of capping mixture, gently agitate on the wrist-action shaker for 15 min and drain.
9. Wash the beads with DMF (3 × 10 mL) and drain.
10. Add the piperidine solution for Fmoc removal (10 mL), shake for 10 min and drain the solid support.
11. Repeat step 10 a second time.
12. Wash the beads with DMF (3 × 10 mL), DCM (3 × 10 mL) and Et_2O (3 × 10 mL) and drain.

Cleavage of the peptide from the solid support:

13. Dry the solid support inside the reactor by connecting a vacuum pump to the lower stopcock.
14. Add a 10 mL cocktail of TFA/H_2O/TIS/thioanisole/thiophenol 87.5/2.5/5/2.5/2.5 (v/v/v/v/v) to the dry solid support. Shake the mixture on a wrist-action shaker for 2 h (*see* **Note §16**).
15. Precipitate the SEA^{on} peptide by adding dropwise the TFA solution from step 14 to an ice-cooled and stirred solution (200 mL) of Et_2O and *n*-heptane (1:1 v/v).
16. Transfer the suspension containing the precipitated SEA^{on} peptide to 50 mL plastic tubes (*see* **Note §17**), centrifuge at 4500 rpm for 5 min and discard carefully the supernatant.
17. Resuspend the pellet in Et_2O (100 mL), vortex the suspension, centrifuge for 5 min and discard carefully the supernatant.
18. Let the peptide dry by placing the open tubes for about 5 min in the fume hood (*see* **Note §18**).

SEA^{on} / MPA exchange reaction:

19. Fill a 1.5-mL microfuge tube with MPA (0.8 mL).
20. Fill a 50 mL plastic tube with deionized water (15 mL)
21. Fill a 1.5-mL microfuge tube with 6 M NaOH (1 mL).
22. Weigh guanidine hydrochloride (10 g) in a 50 mL plastic tube.
23. Introduce into a glove box the cleaved peptide (step 18) as well as all the reagents needed for the SEA^{on} / MPA exchange reaction (steps 19-22) (*see* **Notes §19** and **20**).
24. Into the glovebox, mix MPA (0.625 mL) and water (11.9 mL) in an empty 50 mL plastic tube. Adjust the pH of the solution to 4.0 by adding 6 M NaOH (≈ 400 µL) with a micropipette. Add this solution to the peptide.

25. Add guanidine hydrochloride (≈ 8.6 g) until complete solubilization of the peptide (*see* **Note §21**) and readjust the pH to 4.0 by addition of 6 M NaOH (≈ 250 µL).
26. Let the reaction proceed at 37°C.
27. Monitor the progress of the SEA^{on} / MPA exchange reaction by UPLC-MS analysis (*see* **Note §22**). Collect 5 µL aliquots of the reaction mixture and dilute these samples with 10% AcOH in water (100 µL) prior to UPLC-MS analysis.
28. Once the SEA^{on} / MPA exchange reaction is over (16 h), add AcOH (1.5 mL). Remove the reaction mixture from glovebox and extract the reaction mixture with Et_2O (4 ×10 mL) to remove MPA. Filter the crude solution through a disposable syringe filter.
29. Purify the peptide by preparative HPLC. Use the following elution conditions. Gradient: 0-10% eluent B in 5 min then 10-35% in 30 min, flow rate: 20 mL/min, RT. Analyze the fractions by MALDI-TOF mass spectrometry.
30. Pool the pure fractions in a 50 mL plastic tube, freeze the solution with liquid nitrogen and lyophilize. The bifunctional peptide **2** (25.3 mg, 20% overall from SEA PS solid support) was isolated as a white powder (*see* **Note §23**).
31. Check the identity and purity of the synthesized peptide by UPLC-MS analysis. R_t = 11.24 min. *m/z* = 1766.25 ($[M+2H]^{2+}$), 1177.75 ($[M+3H]^{3+}$), 883.50 ($[M+4H]^{4+}$); calcd. for M (average): 3530.25, found: 3530.82.

SEA^{off} peptide **1** and cysteinyl peptide **3** can be prepared according to the published procedures [17].

3.3 One-pot C-to-N three peptide segment assembly of K1 domain

1. Prepare two 1.5-mL microfuge tubes, each containing 287 mg of Gn·HCl (*see* **Note §24**).
2. Prepare two 1.5-mL microfuge tubes, each containing 16.8 mg of MPAA (*see* **Note §25**).
3. Weigh sodium ascorbate (19.8 mg) in a 1.5-mL microfuge tube (*see* **Note §26**).
4. Weigh TCEP·HCl (28.6 mg) in a 1.5-mL microfuge tube.
5. Weigh SEA^{off} peptide **1** (2.18 mg, 0.608 µmol, 1 equiv.) in a 0.5-mL microfuge tube.
6. Weigh SetCys peptide **2** (2.50 mg, 0.610 µmol, 1 equiv.) in a 0.5-mL microfuge tube.
7. Weigh cysteinyl peptide **3** (2.78 mg, 0.609 µmol, 1 equiv.) in a 0.5-mL microfuge tube.
8. Transfer 1 mL of 0.1 M sodium phosphate buffer (pH 7.2) in a 1.5-mL microfuge tube.

9. Prepare two 1.5-mL microfuge tubes filled respectively with 1 mL of NaOH 6 N and 1 mL of HCl 6 M as well as several 1.5-mL microfuge tubes containing 100 µL of 10% AcOH in deionized water (vol/vol).
10. Cap all the tubes (steps 1-9) and transfer them to the glove box under nitrogen atmosphere (*see* **Notes §19** and **20**).

First ligation: Assembly of the elongated SetCys peptide **4**.

11. Transfer 300 µL of 0.1 M, pH 7.2 phosphate buffer (step 8) to one of the microfuge tubes containing Gn·HCl (see step 1). Vortex until dissolution of the guanidinium salt and transfer this solution to one of the microfuge tubes containing MPAA (see step 2). Adjust the pH of the solution to 7.3 by addition of 6 M NaOH (*see* **Note §27**). The final concentration of MPAA is 200 mM.
12. Transfer 152 µL of the MPAA solution prepared in step 11 to the microfuge tube containing peptide **1** (step 5). Vortex until dissolution of peptide **1** and transfer the solution to the microfuge tube containing peptide **3** (step 7). Vortex until dissolution of peptide **3** and transfer the solution to the microfuge tube containing peptide **2** (step 6). Vortex until dissolution of peptide **2** (*see* **Note §28**). The final concentration of each peptide is 4 mM. Note that the dissolution of peptides **1**, **2** and **3** leads to a decrease of the pH to 6.8.
13. Place the reaction tube in a heating block kept at 37 °C and allow the mixture to react for 4.5 h.
14. Check by UPLC-MS analysis that peptide segments **2** and **3** have been consumed to produce the elongated SetCys peptide **4** and that SEA peptide segment **1** has not reacted. Transfer 3 µL of the reaction mixture to a microfuge tube containing 100 µL of AcOH 10% (vol/vol) in deionized water. Vortex and remove the sample from the glove box. Extract the sample with diethyl ether (4 × 0.5 mL) to remove MPAA (*see* **Note §29**) and add TCEP (3 mg) (*see* **Note §30**). Inject 10 µL of the reaction sample into the UPLC-MS system

Second ligation: Assembly of the biotinylated HGF 125-209 polypeptide **5**.

15. Transfer 300 µL of 0.1 M, pH 7.2 phosphate buffer (see step 8) to the second microfuge tube containing Gn·HCl (see step 1). Vortex until dissolution of the guanidinium salt and transfer the solution to the microfuge tube containing sodium ascorbate (step 3). Vortex until dissolution of sodium ascorbate and transfer the solution to the microfuge

tube containing TCEP (step 4). Vortex until dissolution of TCEP and transfer the solution to the second microfuge tubes containing MPAA (step 2). Adjust the pH of the solution to 5.4 by addition of 6 M NaOH (≈ 60 µL) (*see* **Note §27**). The final concentration of MPAA, TCEP and sodium ascorbate is 200 mM.

16. Transfer 152 µL of the solution prepared in step 15 to the centrifuge tube containing the first ligation mixture (step 13). Adjust the pH of the solution to 5.5 by addition of 6 M HCl (≈ 2.5 µL) (*see* **Note §31**). The final concentrations of MPAA, TCEP and sodium ascorbate are respectively 200 mM, 100 mM and 100 mM.
17. Place the reaction tube in a heating block kept at 37 °C.
18. Monitor the ligation reaction leading to polypeptide **5** by UPLC-MS analysis. Transfer 4 µL of the reaction mixture to a microfuge tube containing 100 µl of AcOH 10% (v/v) in deionized water. Vortex and remove the sample from the glove box. Extract the sample with diethyl ether (4 × 0.5 mL) to remove MPAA. Inject 10 µL of the reaction sample into the UPLC-MS system to confirm that peptide segments **1** and **4** have been consumed to produce the polypeptide **5**.
19. After completion of the reaction (40 h), remove the tube containing the reaction mixture from the glove box.
20. Transfer the content of the reaction tube to a 15-mL plastic tube and add 8 mL of a 7.5% AcOH solution in deionized water (v/v). Extract the mixture with diethyl ether (4 × 5 mL) to remove the MPAA. Filter the crude solution through a disposable syringe filter.
21. Purify the target polypeptide **5** by preparative HPLC. Use the following elution conditions. Gradient: 0-10% eluent B in 5 min then 10-35% in 30 min, 6 mL/min, RT. Analyze the fractions by MALDI-TOF mass spectrometry.
22. Pool the purified fractions in a 50 mL plastic tube, freeze the solution with liquid nitrogen and lyophilize it for 2 days. The reaction yielded the polypeptide **5** (2.05 mg, 29%) as a white powder.
23. Check the purity and identity of the synthesized peptide by UPLC-MS and MALDI-TOF mass spectrometry. **LC-MS analysis**. R_t = 11.40 min. *m/z* = 1666.17 ($[M+6H]^{6+}$), 1428.42 ($[M+7H]^{7+}$), 1250.08 ($[M+8H]^{8+}$), 1111.25 ($[M+9H]^{9+}$), 1000.25 ($[M+10H]^{10+}$), 909.42 ($[M+11H]^{11+}$), 833.75 ($[M+12H]^{12+}$), 769.58 ($[M+13H]^{13+}$), 714.67 ($[M+14H]^{14+}$), 667.17 ($[M+15H]^{15+}$). Calcd. for M (average): 9993.40, found: 9992.14. **MALDI-TOF analysis** (matrix: 2,5-dihydroxybenzoic acid). Calcd. for $[M+H]^+$ (average): 9994.4, found: 9993.1.

4 Notes

Note §1. The synthesis of the SetCys amino acid **6** was repeated several times on gram scale by different experimenters. The yields were quite reproducible.

Note §2. The synthesis of the SetCys amino acid **6** by an experienced chemist requires 3 weeks. Once synthesized, the SetCys residue is stable and can be stored for months in the fridge (4 °C).

Note §3. After extraction, the aqueous layer has to be neutralized with bleach since the reaction with KSeCN leads to the formation of one equivalent of cyanide anion.

Note §4. The addition of $NaBH_4$ leads to active liberation of molecular hydrogen. The excess of gas should be evacuated through an outlet.

Note §5. If aldehyde **10** cannot be used for the next step on the same day, we advise to store it at -20 °C under an argon atmosphere overnight.

Note §6. The addition of K_2CO_3 solution or concentrated HCl to the mixture can induce CO_2 release and bubbling.

Note §7. The pH of the aqueous solution of K_2CO_3 must be at least pH 9 after crude dissolution to best achieve free amine extraction by the organic solvent. If the pH is too low, add more 0.25 M K_2CO_3 aqueous solution.

Note §8. The powdered 3 Å molecular sieve was activated under high vacuum (< 1 mbar), by heating the flask with a heatgun for ~15 min. The flask containing the molecular sieve was filled with argon during cooling to RT.

Note §9. There are strong conformational restrictions in compounds **14**, **15** and **6**. As a consequence, NMR spectra of these compounds at 298 K are complex due to the overlapping of the signals given by all the conformers present in solution. Because no clear description of the NMR spectra can be provided, FID files are given to facilitate the identification of the synthesized compounds by direct comparison. These files can be opened using for example TopSpin® software from Bruker.

Note §10.The presence of the Fmoc group in compound **14** induces conformational restrictions in the molecule. However, variable temperature NMR experiments performed in deutered DMSO show that such conformational barriers can be overcome by heating the sample, leading to the coalescence of NMR signals above 70 °C. Once the selenosulfide bond is formed by iodine oxidation, the conformational restrictions are so strong in the resulting cyclic species (compounds **15** and **6**) that several conformers are detected by NMR in solution, whatever the temperature.

Note §11. For TLC analysis, one drop of the crude mixture is poured in a biphasic mixture containing ~500 µL of a 1 M $Na_2S_2O_3$ solution and ~200 µL of EtOAc. The tube is vigorously

shaken and the upper layer is spotted on the TLC plate against the reference for the starting material.

Note §12. If needed, add brine to improve the separation between aqueous and organic layers.

Note §13. We noticed that evaporation of the crude mixture to dryness before purification systematically resulted in lower isolated yields. Therefore, we recommend to concentrate the mixture to a final volume of 5-10 mL.

Note §14. The silica gel chromatography column is loaded with the solution (final volume of 5-10 mL) obtained after concentrating the crude mixture under reduced pressure.

Note §15. At this stage, we highly recommend to check the completion of the coupling step. Take few polymer beads in order to perform a microcleavage and mass analysis. If coupling is not complete, repeat steps 3-7. Perform microcleavage as described in steps 12-16 using 1 mL of TFA cocktail.

Note §16. The shaking power should be set appropriately to guarantee the mixing, but not too vigorous to avoid the continuous projection of TFA on the reactor stopper. Plastic stoppers are weakly resistant to TFA. Their degradation products may contaminate the peptide-containing cleavage solution.

Note §17. For safety reasons, the centrifuge tubes should be correctly balanced by mass.

Note §18. If the tranthioesterification step is not performed straightaway, dissolve the dried peptide in water with 0.1 % TFA, freeze the solution with liquid nitrogen and lyophilize it.

Note §19. The glove box allows performing transthioesterification as well as ligation experiments in a nitrogen atmosphere with oxygen levels below 10 ppm. Very low oxygen levels are recommended since molecular oxygen oxidizes thiol or selenol species (SEA^{on} thiols, Cys thiol, MPAA …) into unproductive dichalcogenides. This side-reaction cannot be reversed when reactions are performed in the absence of TCEP as for some chemical transformations described in this protocol.

Note §20. The tubes that contain peptide segments, reagents and buffers must be tightly capped during transfer into the glove box, which requires purging the airlock by pumping and filling with nitrogen.

Note §21. Guanidine hydrochloride is a strong peptide denaturant. It was added at the beginning of the SEA^{on} / MPA exchange reaction to improve the low solubility of the SEA^{on} peptide in the MPA solution at pH 4.

Note §22. We noticed that bifunctional peptides such as compound **2** can overreact, leading to the formation of cyclic SetCys peptides by intramolecular ligation. To minimize this side

reaction, the transthioesterification step has to be carefully monitored and stopped immediately after completion.

Note §23. The yield is calculated, based on the quantity of solid support used to elongate the bifunctional peptide **2**.

Note §24. Due to its denaturant properties, guanidine hydrochloride is used as classical additive during ligation. At a 6 M concentration, it avoids any partial folding of the peptide segments that could mask reactive ends and affect ligation efficiency.

Note §25. MPAA catalyzes NCL and SEA ligation processes through the *in situ* formation of an arylthioester peptide segment by a thiol-thioester exchange mechanism. Usually, the aryl thioester peptide derived from the alkyl thioester or SEA peptide reacts quickly with the cysteinyl component so that it is usually not observed in the HPLC traces of the crude ligation mixture.

Note §26. Sodium ascorbate is a strong radical quencher. It avoids the TCEP-induced deselenization side-reaction that converts the SetCys residue into unproductive *N*-ethyl cysteine.

Note §27. Dissolution of high concentrations of TCEP and/or MPAA in the phosphate buffer results in a significant decrease of the pH well below pH 5.5. MPAA is poorly soluble in these conditions. MPAA is highly soluble in water at and above pH 5.5 due to the ionization of its carboxylic acid group. MPAA will therefore solubilize gradually upon addition of NaOH and vortexing. At this stage, a precise control of the pH is not mandatory. However, the addition of NaOH must be done carefully to avoid a significant increase of the pH, that would require the addition of large volumes of HCl in return.

Note §28. Peptides **1**, **2** and **3** must be added in the MPAA solution in the order described in the protocol (**1** -> **3** -> **2**) to avoid the formation of a SetCys cyclic by-product at the beginning of the reaction. In presence of MPAA, the SetCys residue and the thioester group of bifunctional peptide **2** can react, leading to the cyclisation of the peptidic backbone by intramolecular ligation. However, this side-reaction is kinetically disfavored compared to classical NCL between peptides **1** and **2**. The SetCys reactivity is not an issue as long as a competitive cysteine is present in the reaction mixture.

Note §29. The large excess of MPAA used for catalyzing the ligation must be extracted with diethyl ether before running the analytical or the semi-preparative HPLC systems. This arylthiol absorbs strongly at the wavelengths used for the UV detection by the HPLC systems and can co-elute with the target peptide. The efficient extraction of MPAA requires the acidification of

the reaction mixture well below the p*K*a of the aromatic thiol. This is usually achieved by adding 10% AcOH (v/v) to the reaction mixture.

Note §30. By enabling the reduction of all the dichalcogenides that are contained in the sample after the MPAA extraction, the addition of TCEP facilitates the analysis of the ligation mixture. Following this approach, HPLC chromatograms show a single set of peaks corresponding to the reactants and the products of the ligation under their reduced forms.

Note §31. The pH of the reaction was decreased to 5.5 in order to accelerate the SEA ligation between peptide segments **1** and **4**. In the SEA ligation, the peptide thioester which reacts with the cyteinyl peptide to give the ligated product is produced *in situ* from the SEA^{on} peptide by *N*,*S*-acyl shift (Figure 1). Such a rearrangement is pH-dependent and is favored under mild acidic conditions, typically pH 5.5. Decreasing the pH to 5.5 also accelerate the spontaneous decomposition of the SetCys residue into cysteine. Previous studies [13] showed that such a reaction proceeds through an intramolecular substitution of the ammonium group by the selenoate ion (Figure 1). The rate of SetCys decomposition is optimal around pH 5.5-6.0 since the ammonium group and the selenoate ion are the predominant species at this working pH and are respectively the best leaving group and nucleophile for the substitution reaction. We also noticed that the departure of the 2-selenoethyl limb is greatly facilitated by the nearby SetCys thiol, see ref [13].

Acknowledgements

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Figures

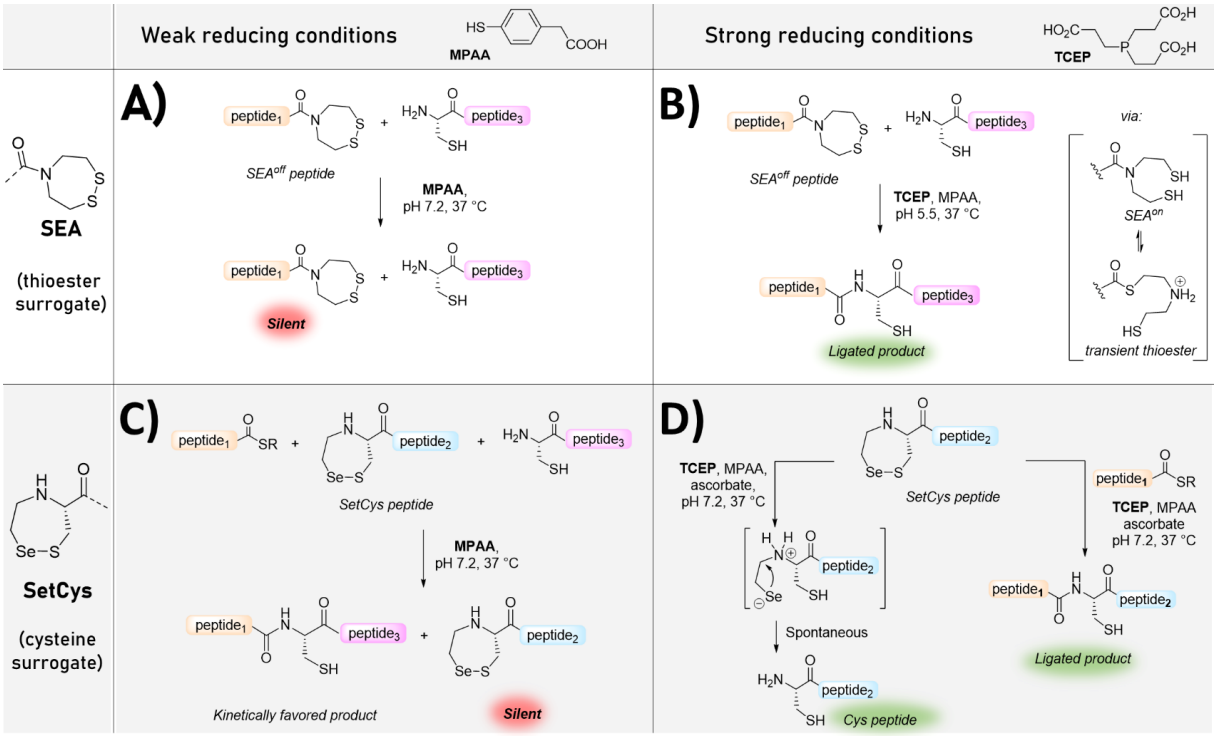

Figure 1. The reactivity of *N*-(2-selanylethyl)cysteine (SetCys) and SEA^{off} peptides is controlled by the nature of the reducing agent present in solution.

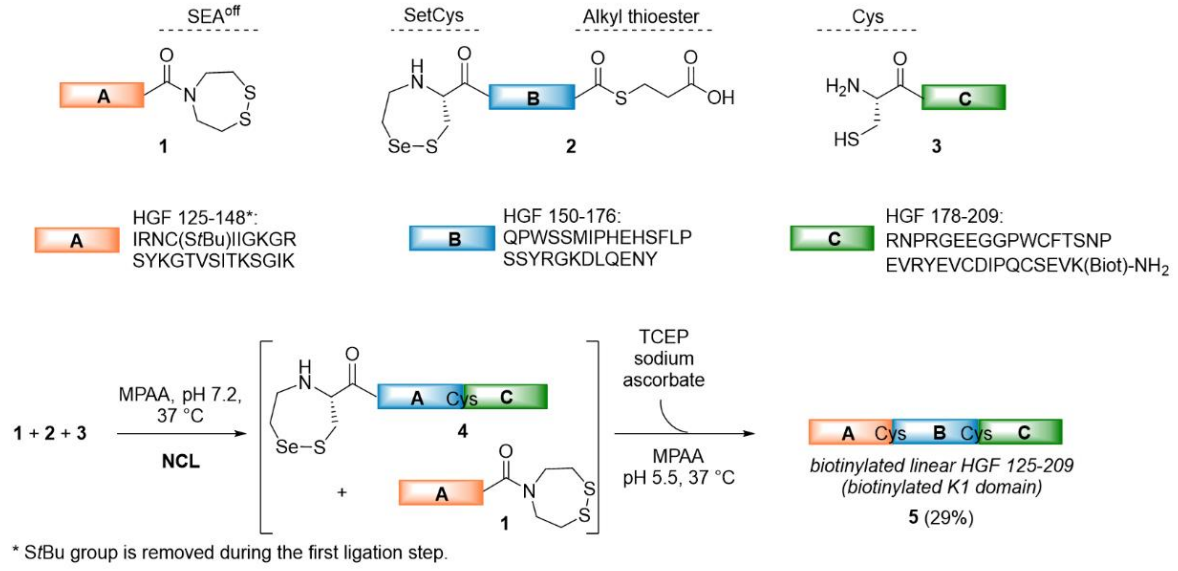

Figure 2. Application of SetCys to the one-pot three peptide segment assembly of proteins is illustrated in this protocol by the total chemical synthesis of biotinylated HGF 125-209 polypeptide, which corresponds to the kringle 1 (K1) domain of HGF.

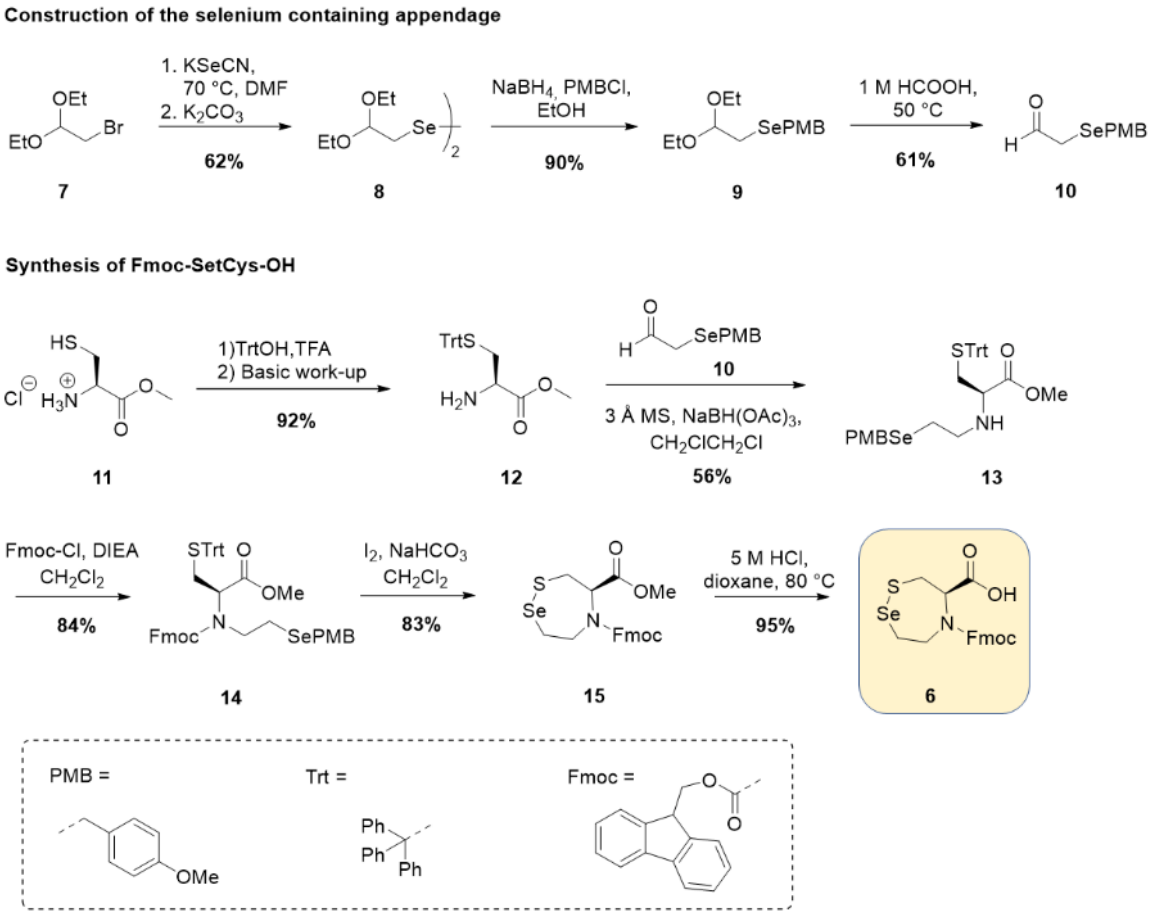

Figure 3. Synthesis of the Fmoc-protected SetCys amino acid **6**.

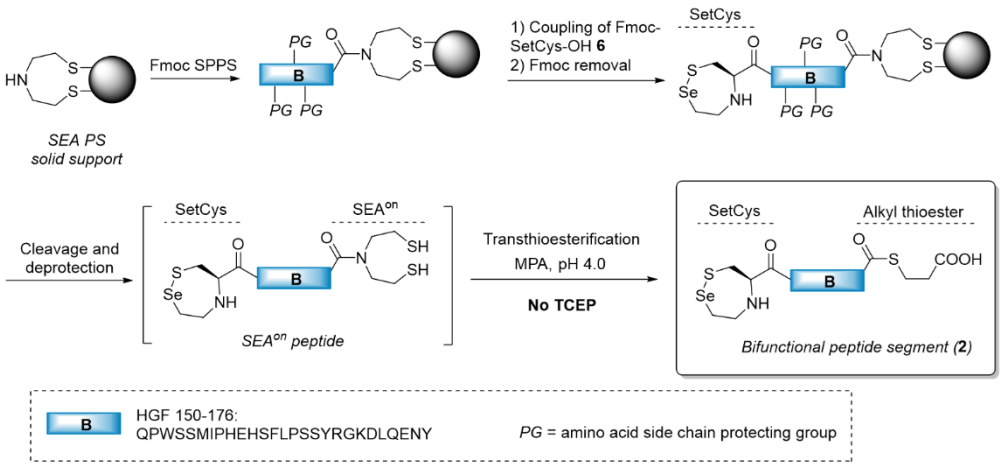

Figure 4. General approach for the preparation of *N*-terminal SetCys peptide thioesters.

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